

Competition of Pyridoxal 5'-Phosphate with Ribulose 1,5-Bisphosphate and Effector Sugar Phosphates at the Reaction Centers of the Spinach Ribulose 1,5-Bisphosphate Carboxylase/Oxygenase

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Ribulose 1,5-Bisphosphate Carboxylase/Oxygenase, Effector Studies, Pyridoxal 5'-Phosphate Inhibition

The stimulation of the carboxylase reaction by effectors of ribulose 1,5-bisphosphate carboxylase/oxygenase displays higher sensitivity towards pyridoxal 5'-phosphate inhibition than the catalytical process itself.

Pyridoxal 5'-phosphate binding to the enzyme is not affected by the modulators 6-phosphogluconate and fructose 1,6-bisphosphate at low concentrations at which these agents stimulate the carboxylation rate.

At higher concentrations these sugar phosphates protect the enzyme against pyridoxal 5'-phosphate inhibition in a similar fashion like the substrate ribulose 1,5-bisphosphate.

Such protection experiments in combination with spectrophotometrical studies of pyridoxal 5'-phosphate binding demonstrate two binding states of ribulose 1,5-bisphosphate at the reaction centers of the enzyme with different requirements for Mg^{2+} . 6-Phosphogluconate functions as protector only in the presence of Mg^{2+} .

Our results imply a competition between pyridoxal 5'-phosphate and substrate or effector sugar phosphates at the reaction centers of the spinach carboxylase. It is proposed that the pyridoxal 5'-phosphate inhibition of the stimulatory activity of these effectors originates from a modification of the regulatory sites of the enzyme caused by pyridoxal 5'-phosphate binding to the catalytical sites.

Introduction

Ribulose 1,5-bisphosphate carboxylases/oxygenases (3-phospho-D-glycerate carboxylase (dimerizing); EC 4.1.1.39) are allosteric enzymes. The activity of these enzymes can be regulated by sugar phosphates, like 6-phosphogluconate and fructose 1,6-bisphosphate, which are structurally related to the substrate RuP_2 . Such agents stimulate the carboxylase and oxygenase reaction at low concentration and inhibit these processes at higher concentrations competitively to RuP_2 [1–7].

The specific functional groups involved in effector binding as well as the mode of action of these compounds at the regulatory sites of the enzyme are still unknown. The subject of this publication is a further characterization and differentiation of the RuP_2 and effector binding to the spinach carboxylase by inhibitor studies with PLP. PLP has been characterized

as an active site directed agent for reactive amino groups of RuP_2 carboxylases/oxygenases [8–11].

Materials and Methods

Materials

Highly purified RuP_2 carboxylase/oxygenase from spinach was prepared as described previously [7, 12].

RuP_2 and the effector sugar phosphates as well as NADPH and PLP were obtained from Sigma. $NaH^{14}CO_3$ and $NaBT_4$ were purchased from Amersham.

Methods

Binding studies with the inhibitor PLP

Spectrophotometrical measurements

The aldimine formation which occurs in the reaction of RuP_2 carboxylase/oxygenase with PLP was detected spectrophotometrically. The absorption change at 432 nm was measured as a function of the PLP concentration similar to the procedure published by Paech *et al.* [9, 10]. A Varian double beam spectrophotometer, model 635 and a Perkin-Elmer

Abbreviations: Bicine, N,N-bis(2-hydroxyethyl)glycine; DTE, dithioerythritol; 2-ME, 2-mercaptoethanol; RuP_2 , ribulose 1,5-bisphosphate; PLP, pyridoxal 5'-phosphate.

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two wavelength spectrophotometer, model 556, was used for these measurements. The following composition of the reaction mixtures was used: 10 mM or 100 mM KOH-bicine-buffer (pH = 8.0); 10 mM Mg²⁺; 0.25 mM EDTA and 1 mM DTE. 0.5–1 mg of the enzyme was added to 2 ml of this mixture. The reaction was started by the addition of PLP and run for at least 20 minutes. For the measurement of the influence of RuP₂ and effector sugar phosphates on PLP binding the enzyme was preincubated with various concentrations of these agents in the presence of 10 mM Mg²⁺ or without Mg²⁺ prior to the addition of PLP.

Fluorimetric binding studies

The reduced enzyme-PLP-complex was detected fluorimetrically after treatment of the aldimine with NaBH₄. The composition of the reaction mixtures essentially corresponded to those applied for the spectrophotometrical experiments. For the fluorimetric binding studies, however, lower enzyme concentrations were used (120–150 µg per 2 ml). The enzyme-PLP-complex was reduced by the addition of NaBH₄ for one hour. A hundredfold excess of the reducing agent over the PLP concentration was used. After the reduction the samples were exhaustively dialyzed against 0.1 M KOH-bicine-buffer (pH = 8.0) with addition of 5 mM 2-mercaptoethanol (2-ME).

The fluorescence of the reduced enzyme-PLP-complex was measured with a Hitachi/Perkin-Elmer MPF 4 spectrofluorimeter in ratio mode. The excitation wavelength was 328 nm. Fluorescence emission was detected at 390 nm, the emission maximum of the complex.

Tritium incorporation into the enzyme-PLP-complex

The reaction mixtures were the same as used for the fluorimetric binding studies. The Schiff base formed by the enzyme with PLP was reduced with NaBH₄. Thereafter the samples were exhaustively dialyzed against solutions of 50 mM NaCl with addition of 5 mM 2-ME. After the dialysis the tritium labelled enzyme was detected by liquid scintillation spectrometry.

Assays for the carboxylase activity

Assays for the measurement of the carboxylase activity were performed essentially as described in ref. [7].

a) General assay: The reaction mixture for the determination of the carboxylase activity contained either 10 mM or 100 mM KOH-bicine buffer (pH = 8.0); 20 mM NaH¹⁴CO₃ (0.1–0.5 µCi/µmol); 20 mM MgCl₂; 0.5 mM RuP₂; 0.1 mM EDTA and 1 mM DTE. The final volume was 0.5 ml. The enzyme was preincubated in the reaction mixture without RuP₂ for 10 minutes at 25 °C. The reaction was initiated by the addition of RuP₂ and stopped after 1 minute by acidification with 0.2 ml 2 N HCl. Aliquots of the reaction mixture are dried and the acid stable products were determined by scintillation counting.

b) Assay in the presence of effectors: The enzyme was preincubated for 20 minutes in the presence of the effectors. The composition of the preincubation mixtures is specified under Legends. The bicarbonate concentration in these experiments was 0.1 mM.

The reaction was started by the addition of either RuP₂ or a mixture of RuP₂ and NaH¹⁴CO₃. The reaction time was 5 minutes at 25 °C. The samples were treated further as outlined under general assay.

Protection of the RuP₂ carboxylase/oxygenase against PLP deactivation by preincubation with RuP₂ and effector sugar phosphates

The carboxylase was preincubated in 100 mM KOH bicine-buffer (pH = 8.0); 0.2 mM EDTA and 2 mM DTE with various concentrations of RuP₂ or the effector 6-phosphogluconate either in the presence of 10 mM MgCl₂ or without MgCl₂ for 20 minutes. Afterwards PLP was added in a concentration of 0.5 mM followed by a second 20 minute incubation. Finally the samples were reduced with NaBH₄ (0.1 M) for one hour and exhaustively dialyzed against the reaction buffer. Aliquots of the reaction mixture were assayed for carboxylase activity, as described in ref. [7]. For control aliquots of the enzyme were reduced in the same way after incubation without sugar phosphate and the inhibitor PLP.

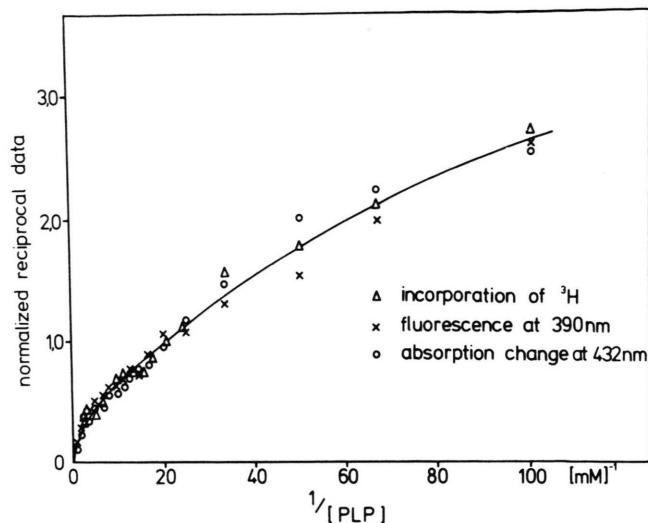
All experiments were performed at 25 °C.

Results

Binding studies were performed with PLP, as demonstrated in Fig. 1.

a) The absorption change at 432 nm which is characteristic for the aldimine formation between the spinach enzyme and PLP;

Fig. 1. Double reciprocal diagram for the binding of PLP to the spinach carboxylase a) Absorption change at 432 nm (○); b) fluorescence of the reduced carboxylase-PLP-complex (×) and c) tritium incorporation into the carboxylase-PLP-complex by reduction with NaBT₄ (△) as a function of the reciprocal PLP concentration. All data were normalized on the corresponding result at a PLP concentration of 0.05 mM. Experimental conditions as described under Methods.



b) the fluorescence emission at 390 nm of the reduced carboxylase-PLP complex and

c) the tritium incorporation into this complex after the reduction of the primarily formed Schiff base with NaBT₄ were determined as a measure of binding of PLP to the RuP₂ carboxylase/oxygenase from spinach.

An excellent agreement of the binding patterns for all three methods was obtained. Our results essentially confirm the findings of Paech *et al.* [9, 10]. Assuming an extinction coefficient $\epsilon = 5800 \text{ M}^{-1} \text{ cm}^{-1}$ for the Schiff base formation between the carboxylase and PLP published by these authors [9] we found that 1.5–2 mol of PLP were bound per functional unit AB of the enzyme. From sodium dodecyl sulfate/polyacrylamide gel electrophoresis of our tritium labelled carboxylase-PLP-complexes it is apparent that 95% of the inhibitor is localized in the large subunit A of the enzyme. Only a minor incorporation (approx. 5%) was observed in the small subunit B [13].

In Figs 2 and 3 the effect of PLP on the carboxylase activity of the spinach enzyme was studied under various reaction conditions. Fig. 2 shows the influence of Mg²⁺ and the effector 6-phosphogluconate on the PLP inhibition of CO₂ fixation.

In the absence of the inhibitor the effector 6-phosphogluconate enhances the carboxylation rate by a factor of 3.5, if Mg²⁺ is added to the preincubation mixture. The effector activity of 6-phosphogluconate, which is defined as the difference in the car-

boxylation rates in the presence and absence of the effector was 250%. If Mg²⁺ is omitted from the preincubation mixture, no stimulation of the CO₂ fixation by 6-phosphogluconate is observed.

In Fig. 2 the data for each curve were normalized on the carboxylation rate measured under the different reaction conditions in the absence of PLP. It is apparent that in the presence of 10 mM MgCl₂ CO₂ fixation is stimulated at low PLP concentrations in the range of 0.1–20 μM . The maximal enhancement effect was about 30% of the carboxylation rate

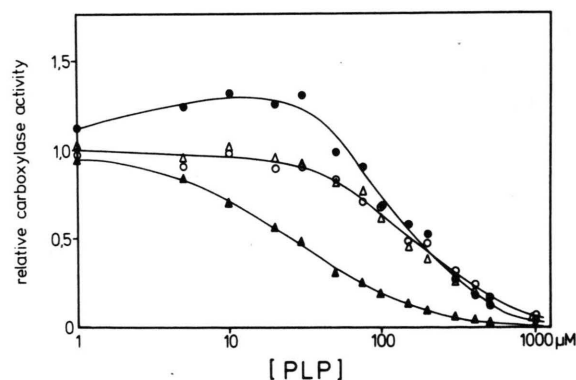


Fig. 2. Relative carboxylase activity as a function of the PLP concentration under various preincubation conditions. The spinach carboxylase was preincubated for 20 minutes in 0.1 M KOH-bicine-buffer (pH = 8.0); 0.1 mM EDTA; 1 mM DTE and a) with 10 mM MgCl₂ (●); b) without MgCl₂ (○); c) with 10 mM MgCl₂ and 20 μM 6-phosphogluconate (▲) and d) with 20 μM 6-phosphogluconate without MgCl₂ (△). Afterwards the carboxylase reaction was started by the addition of 0.5 mM RuP₂ and 0.1 mM NaH¹⁴CO₃. The reaction time was 5 minutes.

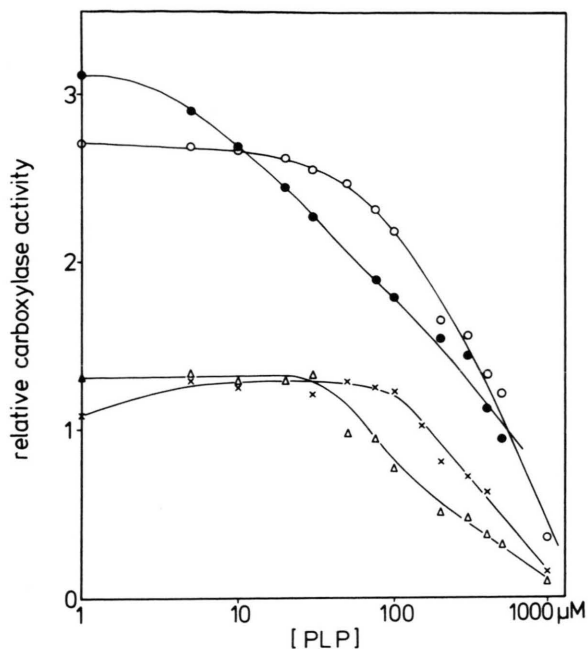
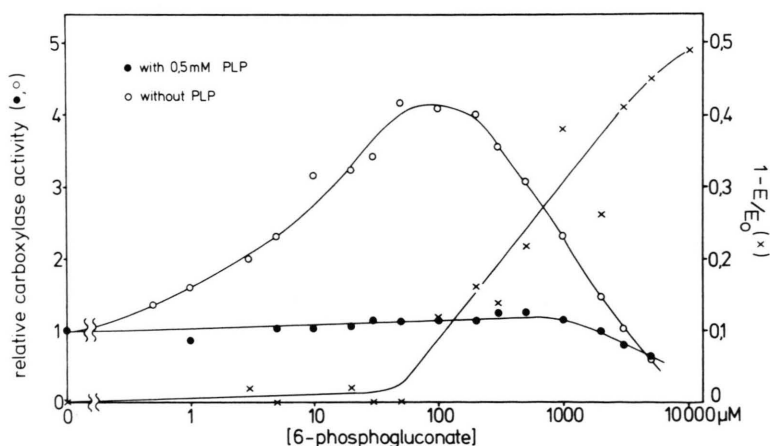


Fig. 3. Relative carboxylase activity as a function of the PLP concentration. The enzyme was preincubated for 20 minutes in 0.1 M KOH-bicine-buffer (pH = 8.0); 0.1 mM EDTA; 1 mM DTE; 10 mM MgCl₂ and 0.1 mM NaH¹⁴CO₃ and a) without effector (x); b) with 20 μM 6-phosphogluconate (●); c) with 200 μM fructose 1,6-bisphosphate (Δ); d) with 1 mM NADPH (○). The reaction was started by the addition of 0.5 mM RuP₂. Reaction time: 5 minutes.

without PLP. Stimulation is not observed, if Mg²⁺ is omitted from the preincubation mixture. At higher PLP concentrations (20 μM–1 mM) PLP functions as an inhibitor of the carboxylase reaction both in the presence of 10 mM Mg²⁺ as well as without Mg²⁺. 50% inhibition is obtained at about 150 μM PLP.

Fig. 4. Relative carboxylase activity (○, ●) and relative decrease of the absorption change of the carboxylase-PLP-complex at 432 nm (x) as a function of the 6-phosphogluconate concentration in the preincubation medium. 1. Assays: The enzyme was preincubated for 20 minutes in 0.1 M KOH-bicine-buffer (pH = 8.0); 0.1 mM EDTA; 1 mM DTE; 10 mM MgCl₂ and various effector concentrations. a) with 0.5 mM PLP (●) and b) without PLP (○). The reaction was started by the addition of 0.5 mM RuP₂ and 0.1 mM NaH¹⁴CO₃. 2. Spectrophotometrical measurements as indicated under Methods.



In the presence of 10 mM Mg²⁺ in the preincubation the PLP inhibition of the carboxylase activity is enhanced at low concentrations of the effector 6-phosphogluconate (20 μM). Effective inhibition occurs already at PLP concentrations below 10 μM under these conditions. 50% inhibition is observed at about 25 μM. If Mg²⁺ is omitted in the preincubation no enhancement of the PLP inhibition is detectable and the same inhibition profile is obtained as without the effector.

In Fig. 3 the PLP inhibition of CO₂ fixation in the presence of 6-phosphogluconate, fructose 1,6-bisphosphate and NADPH is compared with the inhibition without effector. The effector activities of these agents in the absence of PLP were 227% for 6-phosphogluconate, 38% for fructose 1,6-bisphosphate and 178% for NADPH.

In Figs 4 and 5 the effector activities of 6-phosphogluconate and fructose 1,6-bisphosphate in the carboxylase reaction have been determined in the presence of 0.5 mM PLP and without the inhibitor. The stimulation of the carboxylase activity by these regulatory sugar phosphates is always more strongly inhibited by PLP than the catalytical process. The inhibitory effect of 6-phosphogluconate and fructose 1,6-bisphosphate on CO₂ fixation is shifted to higher sugar phosphate concentrations in the presence of PLP. These measurements have been correlated to the decrease of PLP binding in the presence of these agents indicated by the absorption change at 432 nm.

In Figs 6 and 7 the protective effect of the substrate RuP₂ and the effector 6-phosphogluconate on the carboxylase activity are demonstrated. Different Mg²⁺ dependent protection effects have been obtained for these sugar phosphates.

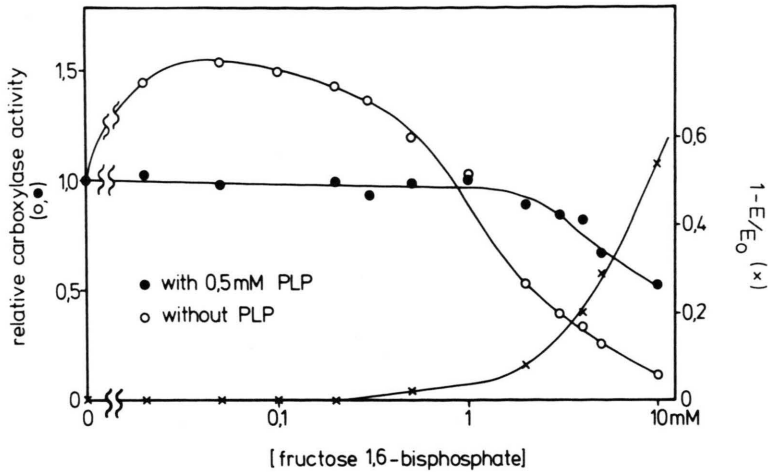


Fig. 5. Relative carboxylase activity (○, ●) and relative decrease of the absorption change of the carboxylase-PLP-complex at 432 nm (x) as a function of the fructose 1,6-bisphosphate concentration in the preincubation medium. Experimental conditions as described in the legend of Fig. 4.

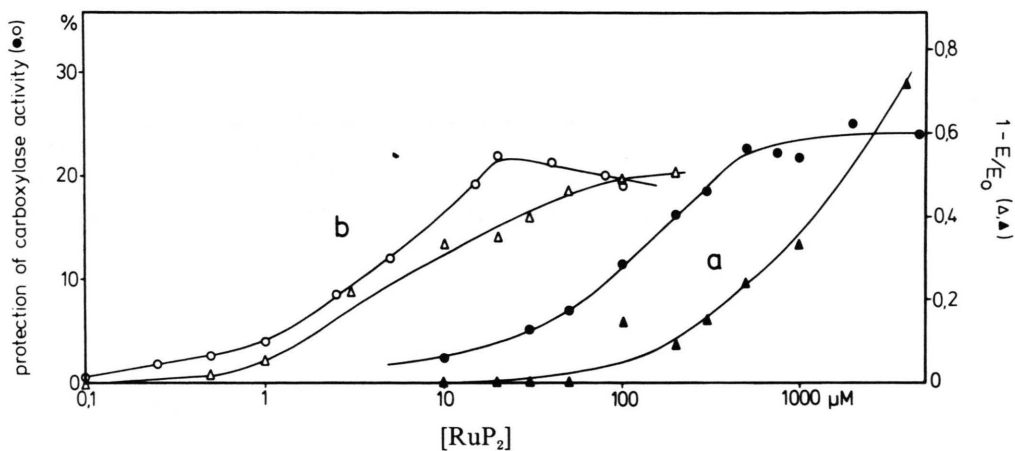


Fig. 6. Protection of the spinach carboxylase against PLP-inhibition (○, ●) and relative decrease of the absorption change of the carboxylase-PLP-complex at 432 nm (▲, △) as a function of the RuP₂ concentration in the preincubation medium. a) in the presence of 10 mM MgCl₂ (●, ▲) and b) without MgCl₂ (○, △) in the preincubation mixture. Experimental conditions as indicated under Methods.

RuP₂ functions as an effective protector of the carboxylase both in the presence of 10 mM MgCl₂ and without Mg²⁺ in the preincubation medium. In the presence of Mg²⁺ protection is found in a range of 10 μM–1 mM RuP₂, whereas in the absence of Mg²⁺ the protective effect of RuP₂ is observed at lower concentrations between 0.5 and 10 μM.

Similar results have been obtained for the RuP₂ induced decrease of the absorption change at 432 nm characteristic for the aldimine formation between

reactive amino groups of the carboxylase and PLP. In the absence of Mg²⁺ the absorption change is reduced to maximally 45% at RuP₂ concentrations between 1 and 100 μM, while in the presence of 10 mM Mg²⁺ a decrease of more than 80% was found in a concentration range of 0.05–4 mM RuP₂. Different carboxylase preparations were used for the protection studies and the spectrophotometrical measurements. A lower PLP concentration (100 μM) was used in the optical measurements than in the

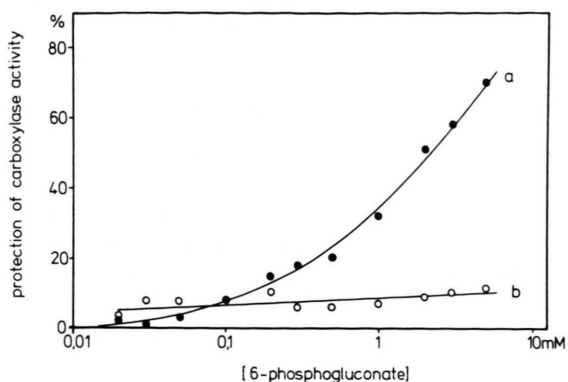


Fig. 7. Protection of the spinach carboxylase against PLP-inhibition by preincubation with 6-phosphogluconate. a) in the presence of 10 mM MgCl₂ (●) and b) without MgCl₂ (○) in the preincubation mixture. Experimental conditions as indicated under Methods.

protection experiments (500 μM) for instrumental reasons.

In contrast to the substrate RuP₂ the effector 6-phosphogluconate shows a significant protection of the carboxylase reaction against PLP inhibition only in the presence of Mg²⁺.

Discussion

In this publication the action of PLP on the carboxylase reaction was studied under various reaction conditions. The special interest in this work was to detect differences in the mode of binding of RuP₂ and effector sugar phosphates, like 6-phosphogluconate and fructose 1,6-bisphosphate, at the catalytical and regulatory centers of the RuP₂ carboxylase/oxygenase from spinach.

PLP inhibits both the carboxylase and the oxygenase reaction [9] and has been reported as an active site directed agent for reactive amino groups of RuP₂ carboxylases/oxygenases [9–11].

Inhibitor studies with PLP under various reaction conditions are shown in Figs 2–5. From Fig. 2 it is apparent that PLP inhibition of the carboxylase is markedly enhanced by the effector 6-phosphogluconate only in the presence of Mg²⁺, consistent with the Mg²⁺ requirement of the effector binding [7, 12]. A conformational change of the enzyme is induced under these conditions, as detected fluorimetrically [7, 12] which obviously causes a tighter binding of the inhibitor PLP.

Whitman *et al.* [11] observed an analogous enhancement effect by activation of the *Rhodospirillum rubrum* enzyme with Mg²⁺ and bicarbonate. Similar results we have obtained also for the spinach enzyme under these conditions (data not shown). From these experiments it follows that PLP inhibition of the carboxylase reaction is most effective, if the enzyme is in an activated state which can be achieved either by bicarbonate or effector activation of the carboxylase in the presence of Mg²⁺. PLP binding therefore can be taken as an indicator for the activation state of the enzyme.

Whitman *et al.* [14] have shown that PLP also functions as an effector of the carboxylase reaction at low PLP concentrations. Our experiments confirm their results, as it is apparent from Figs 2 and 3. In contrast to these authors, however, we find a Mg²⁺ requirement for this effect similar to the effector activity of the regulatory sugar phosphates 6-phosphogluconate and fructose 1,6-bisphosphate.

The effect of PLP on the activity of these agents relating to CO₂ fixation was investigated in Figs 3–5 and compared to the action of these compounds on PLP binding. The stimulation of the carboxylase reaction induced by these effectors is inhibited by PLP at lower concentrations than the catalytical process. This effect is most pronounced for 6-phosphogluconate. For example, the effector activities of 6-phosphogluconate and fructose 1,6-bisphosphate are completely abolished at a PLP concentration of 0.5 mM, whereas CO₂ fixation still functions at an appreciable extent (30–40%).

This result could be interpreted as the consequence of a competition between the effectors and PLP at the regulatory sites of the RuP₂ carboxylase/oxygenase. Under these conditions one should expect a decrease of PLP binding with increasing effector concentration in the range, where 6-phosphogluconate and fructose 1,6-bisphosphate express their stimulating effect in the carboxylation reaction (0.1–50 μM for 6-phosphogluconate and 5–200 μM for fructose 1,6-bisphosphate), because it is apparent that these agents form kinetically stable complexes with the enzyme [3]. Our spectrophotometrical binding studies demonstrate that PLP binding is affected not at all by these sugar phosphates under these conditions.

A decrease of the aldimine formation between the carboxylase and PLP, however, is observed at higher concentrations of 6-phosphogluconate and fructose

1,6-bisphosphate, where these agents inhibit CO₂ fixation by competition with RuP₂ at the reaction centers of the enzyme [3, 7]. These results imply a competition between PLP and these sugar phosphates at the catalytical sites of the carboxylase. This interpretation is supported by protection experiments.

The substrate RuP₂ functions as an effective protector against PLP inhibition both in the presence of 10 mM MgCl₂ and without Mg²⁺. In the presence of Mg²⁺ protection is found at essentially higher RuP₂ concentrations than in the absence of Mg²⁺. These protection experiments are in accordance with the effect of RuP₂ on PLP binding, as measured spectrophotometrically. A decrease of the aldimine formation by preincubation of the enzyme with RuP₂ is observed in the presence of 10 mM MgCl₂ as well as without Mg²⁺ in similar ranges of the RuP₂ concentration, as measured previously in the functional studies. These results indicate two binding processes for the substrate RuP₂ with different requirement for Mg²⁺. This is also apparent from other effects which will be presented in a forthcoming publication. These results are consistent with the conclusions of Whitman and Tabita [11] that PLP acts at the RuP₂ binding site of the catalytical center, as demonstrated for the *Rhodospirillum rubrum* enzyme. In contrast to the results of Paech *et al.* [9] and Whitman *et al.* [11] we found that 6-phosphogluconate also functions as an efficient protector against PLP deactivation of the carboxylase. In this case protection occurs only in the presence of Mg²⁺. This again

proves the Mg²⁺ dependence of effector binding. The protection effect is observed at 6-phosphogluconate concentrations between 100 μM and a few mM characteristic for the 6-phosphogluconate binding to the reaction centers of the carboxylase [3, 7].

The results of this paper imply a competition between PLP with RuP₂ and regulatory sugar phosphates at the reaction centers of the spinach carboxylase. From our experiments it follows that PLP does not interfere directly with the allosteric sites of the enzyme. PLP binding at the catalytical sites obviously induces a modification of the effector binding at the regulatory sites. This conclusion points to a difference in the requirement for or the reactivity of functional amino groups for the binding of 6-phosphogluconate and fructose 1,6-bisphosphate at the catalytical and regulatory centers of RuBP carboxylases/oxygenases.

To what extent reactive amino groups for the binding of CO₂ are involved in these processes, as suggested by Paech *et al.* [10], must be clarified by a further analysis of PLP inhibition and substrate binding. Such experiments as well as a further characterization of the effector binding sites of the RuP₂ carboxylase/oxygenase are in progress.

Acknowledgements

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